Genome-wide transcriptome analysis of hydrogen production in the cyanobacterium Synechocystis: Towards the identification of new players

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GENOME-WIDE TRANSCRIPTOME ANALYSIS OF HYDROGEN PRODUCTION IN *SYNECHOCYSTIS*: TOWARDS THE IDENTIFICATION OF NEW PLAYERS

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ABSTRACT

We report the development of new and robust tools for facile integration and meaningful representation of the high throughput data acquired during the study of the genome-wide responses to environmental challenges or genetic modifications of the best-studied cyanobacterium *Synechocystis* sp. PCC6803, which has the potential for the photo-production of sustainable bio-fuels such as hydrogen. These new tools comprise (i) the new design of pan-genomic oligonucleotide DNA microarray to monitor the transcriptome responses of not only the circular chromosome (3.57 Mb; 3,317 genes) but also the 7 plasmids (0.38 Mb; 408 genes altogether), and (ii) the SVGMapping a computational method to map *omic* experimental data onto custom-made templates, depicting metabolic pathways, cellular structures and/or biological processes. We validated our tools, through the identification and representation of the global transcriptome changes triggered by the deletion of *abrB2*, the gene we recently found to encode the repressor of the hydrogenase *hox* operon directing the production of hydrogen. We show here, for the first time, that AbrB2 is a master regulator that regulates (mostly negatively) a large number of chromosomal genes involved in regulation, metal transport and protection against oxidative stress, as well as numerous plasmid genes of as yet unknown function. That AbrB2 regulates 12 regulator genes in addition to the *hox* operon suggesting that the regulation of hydrogen production might be more complex than anticipated a hypothesis currently tested in our laboratory. Furthermore, our data suggest that plasmids carry several genes of unknown function, which have been overlooked so far, are involved in hydrogen metabolism, among other important cellular processes mentioned above. Consequently, we believe that the present report will stimulate both basic and applied researches on plasmid functions and their likely relation with hydrogen production.

1. INTRODUCTION

Energy production and consumption has become a major environmental issue [1]. In this context, cyanobacteria, the only known prokaryotes capable of oxygenic photosynthesis, are receiving a growing attention for the sustainable production of the clean fuel hydrogen, due to their (i) simple nutritional requirements, (ii) robustness, (iii) metabolic plasticity, and (iv) the powerful genetics of some model strains. This is true of the unicellular cyanobacterium *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) which possesses a small genome (about 4 Mb; Table 1), comprizing on circular chromosome and seven plasmids (CyanoBase: http://genome.kazusa.or.jp/cyanobase; [2, 3], easily manipulable [4-7]. The pentameric hydrogenase enzyme (HoxEFUYH) is encoded by a heptacistronic operon, which also contains three open reading frames of unknown function (*sll1222*, *ssl2420* and *sll1225* in CyanoBase). The active Hox enzyme, matured by the HoxW

protease and assembled using the six-subunits HypABCDEF complex [8-12] produces H_2 through the reversible reaction $2H^+ + 2\ e^- \leftrightarrow H_2$ (with a bias to H_2 production) [13, 14], which uses NAD(P)H as the source of electrons originating from photosynthesis and/or sugar catabolism, as well as a nickel-iron cluster and several iron-sulfur centers as redox cofactors [14].

Besides the assembly and activity of the Hox enzyme is also important to thoroughly study the mechanisms controlling the expression of the *hox* operon, in order to better understand the role of the Hox enzyme in the global metabolism of the cell, and possibly develop novel tools to enhance the photoproduction of H_2. The *hox* operon is controlled by at least three transcription factors, the LexA (Sll1626) and AbrB1 (Sll0359) proteins acting positively [15, 16] and the AbrB2 factor (Sll0822 in CyanoBase) acting negative [11]. Indeed we recently found that AbrB2 represses the transcription of the *hox* operon through binding to the *hox* operon promoter [17]. Mutants totally or partially depleted of any of these regulatory proteins were analyzed with DNA microarray, which however were not pan-genomic in covering (at most) only 2,852 chromosomal genes out of 3,317 (about 85%) and none of the 408 plasmidic genes of as yet unknown functions [11, 15, 18]. Consequently, we developed truly pan-genomic (oligonucleotide) DNA microarray to truly study the genome-wide transcriptome responses to environmental stresses and/or analysis of *Synechocystis* strains engineered for the production of biofuel. In addition we have developped SVGMapping an R package to facilitate the analysis of the large number of *omics* data and visualize them onto custom-made templates depicting genome organization, metabolic pathways and cellular structures. Using these tools we characterized and compared the transcriptome profiles of our *ΔabrB2* deletion mutant, with an increased level of hydrogen production [17], by comparison with the WT strain. We report AbrB2 is a master regulator that regulates (mostly negatively) a large number of chromosomal genes operating in regulation, metal transport and protection against oxidative stress, as well as numerous plasmid genes of as yet unknown function. These findings suggest that the regulation of hydrogen production might be rather complex, and that plasmid encoded functions, which have been overlooked so far, are involved in hydrogen production.

2. MATERIAL AND METHODS

Using sequentially the Earray and OligoArray softwares (https://earray.chem.agilent.com and http://berry.engin.umich.edu/oligoarray2_1) we designed new and truly pan-genomic oligonucleotide DNA microarray containing a relevant part of the protein coding sequence (CS) of all chromosomal and plasmidic genes (Tran*Syn*6803 project). Following the classical strategy, we used three, two or one 60-mer oligos, depending on the CS length: ≥ 500 bp; 500 to 120 bp; ≤ 120 bp. We performed three independent biological replicates in exponential growth and RNA isolation as we previously described [18] for our mutant *ΔabrB2* [17] and the wild type (WT) strain. One dye swap was carried out for each biological replicate. RNA isolation was performed by RNeasy Midi kit (Qiagen), cDNA synthesis by reverse transcriptase superscript II kit, dNTP and aminoallyl-dUTP (Invitrogen), cDNA purified with by QIAquick PCR Purification kit (Qiagen), tagging with Cy™Dye Post-labelling Reactive Dye Pack (Life Sciences), and synthesis of DNA microarray, hybridization and scans were accomplished by using Agilent Technologies.

Microarray signals and data were analyzed with the Limma package of the R/Bioconductor software [19]. Microarray spot intensities were normalized by subtracting the background and using the LOWESS method with the smooth parameter set to 0.33 as recommended [20]. Normalized measures served to compute the ratios of Cy3/Cy5 intensity and the associated log2-transform (denoted log2-ratios) for each gene. Then, to identify genes with log2-ratios significantly different between the mutant and WT strain, p-values were calculated for each gene using a moderated *t-test* based on an empirical Bayes

analysis, which is equivalent to shrinkage (or expansion) of the estimated sample variances towards a pooled estimate, thereby resulting in a more stable inference. p-values were adjusted for multiple testing [21]. Then, the SVGMapping method was used to integrate and visualize the changes in expression of the responsive genes in their metabolic pathways, cellular function or genome environments.

3. RESULTS AND DISCUSSION

3.1. AbrB2 behaves mostly as a negative regulator

The expression pattern of total mRNA from our recently constructed *abrB2*-deleted mutant (*ΔabrB2*) showing an increased hydrogen production level [17] was compared with the WT strain, using our new DNA glass microarray carrying 3,639 of the 3,725 genes (protein coding sequences) of *Synechocystis*. Using the standard criteria for selection (fold change of mRNA abundance (FC) higher than the absolute value of 2.0, and p-value lower than 0.01%) we found a large number of genes (333; i.e. 8.9% of all the 3,725 genes of *Synechocystis*) to be deregulated in response to the deletion of *abrB2* (Table 1). The highest number of the AbrB2-responsive genes is coding for hypothetical proteins (103 genes) or unknown proteins (134 genes), emphasizing that much is still to be done in the field of functional genomics to unravel the function of uncharacterized genes. Also interestingly, most of the AbrB2-responsive genes (299 out of 333, i.e. 89%) were found to be up-regulated (some of them quite strongly: FC > 9) in the AbrB2-deleted mutant. Together these findings show that AbrB2 is an important regulator, which mostly behaves as a negative regulator in *Synechocystis* growing under standard photoautotrophic conditions.

3.2. AbrB2 regulates (negatively) the *hox* operon encoding the hydrogenase enzyme complex, but neither the *hoxW* gene nor the *hyp* genes operating in the maturation and assembly of the Hox enzyme complex

We found that AbrB2 negatively regulates all the eight-genes of the *hox* operon: *hoxE* (*sll1220*), *hoxF* (*sll1221*), *sll1222*, *hoxU* (*sll1223*), *hoxY* (*sll1224*), *ssl2420*, *sll1225* and *hoxH* (*sll1226*), with a FC comprised between 3.82 and 4.77 (Fig. 1). This finding is consistent with (i) the analysis of an abrB2-less mutant constructed in the glucose tolerant mutant genetic background with DNA microarray not totally pan-genomic [11](we constructed our abrB2-less mutant in the wild-type genetic background, because the WT strain is the organism that actually occurs in Nature, and we designed true pan-genomic DNA microarray to analysis transcriptome profiles); and (ii) our recent gel shift analysis and promoter assay of the AbrB2 fixation on the *hox* operon promoter, which showed that AbrB2 represses the *hox* operon by binding onto its promoter [17]. By contrast, we found that AbrB2 regulates neither *hoxW*, the gene encoding the protease that cleaves a 24-amino-acid peptide from HoxH subunit prior to its assembly into the NiFe-hydrogenase complex [8], nor the *hyp* genes (*slr1675*, *sll1432*, *sll1078*, *sll1079*, *ssl3580*, *slr1498* and *sll1462* coding for $HypA_1B_1A_2B_2CDE$ respectively) which operate in Hox assembly [9]. Collectively, our results show that AbrB2 negatively regulates the *hox* operon, in agreement with our confirmatory data [17] showing that AbrB2 actually represses the *hox* operon through binding onto its promoter. Furthermore, we show here that AbrB2 does not regulate the genes *hoxW* and $hypA_1B_1A_2B_2CDE$ involved in the maturation and assembly of the hydrogenase enzyme complex, to the single exception of *hypF* gene (*sll0322*), encoding a carbamoyl phosphate-converting enzyme [22], which we found to be negatively regulated by AbrB2.

3.3. AbrB2 regulates neither carbon nor nitrogen metabolism

We found that AbrB2 regulate neither the *abrB1* gene (*sll0359*) encoding an activator of the *hox* operon nor the carbon metabolism genes (Fig. 1), in agreement with what found with the non pan-genomic DNA microarray analysis of the abrB2-less mutant created in the glucose tolerant genetic background [11, 16]. In contrast to the previous report, we did not find the *urtA*, *amt1*, *glnB* and *sigE* genes and the *nrtABCD* operon, which operate in nitrogen regulation and assimilation, to be regulated by AbrB2. This discrepancy might somehow result from the fact that our AbrB2-deleted mutant grows as healthy as the WT strain, whereas the abrB2-deleted mutant constructed by Ishii and co-workers, in the glucose tolerant genetic background which possesses several specific mutations as compared to the WT strain [23] exhibits a slow growth. Further experiments will be performed in our laboratory to study the influence of the growth conditions (growth speed) on the transcriptome response of our *abrB2* deleted mutant.

3.4. AbrB2 regulates several transport systems (Fe, S, Co, P, Zn)

We looked with great interest the possible influence of AbrB2 on the expression of the wealth of transport genes because the Hox enzyme uses a Ni-Fe redox center and several Fe-S redox clusters to produce hydrogen, the level of which is known to be affected by Ni, Fe, S, N, C availabilities, positively (Fe, Ni and S) and negatively (C and N) [24-26]. In the case of nitrogen and carbon, theirs limitation decrease the consumption of electrons, which can then be re-directed to and use for hydrogen production. As anticipated, we found that AbrB2 regulates several of the genes operating in the following transport systems (Fig. 1): *cysAPUW* (SO_4^{2-}), *pstABCS* (PO_4^{2-}), *fecBCD* (Fe^{2+}), *ziaABR* (Zn^{2+}), *coaRT and hupE* (Co^{2+}) [27], *kdpABCD* (K^+). These findings prompt us to study further the influence of AbrB2 on the tolerance to Fe, Ni, and S (starvation and excess) stresses. By contrast, we observed no influence of AbrB2 on the expression of the genes encoding the *cbiMOQ* transport systems for Ni (Fig. 1).

3.5. AbrB2 regulates (negatively) the anti-oxidant quinol oxidase and nitric oxide reductase genes but not the photosynthesis genes

Because our *abrB2*-deleted mutant exhibits a normal healthy growth and typical blue-green color under standard photoautotrophic laboratory conditions (data not shown) we anticipated that the expression of the large number of genes involved in photosynthesis would be unaltered. As expected, this was true for genes encoding for the following photosynthesis sub-complexes: photosystem I (PSI), photosystem II (PSII), cytochrome b6/f complex (Cytb6/f), cytochrome c oxidase (Cytc), cytochrome bd-quinol oxidase (Qox), plastocyanin (PC), plastoquinone (PQ), and the associated redox complexes ATP synthase, ferredoxins, FNR (ferredoxin: NADP reductase) and NADH dehydrogenase type-1 and type-2 (Ndh1 and Ndh2). By contrast, we found that AbrB2 negatively regulates the *cydA* (*slr1379*) and *cydB* (*slr1380*) genes (Fig. 1) encoding the cytochrome bd-quinol oxidase, which can reduce O_2 thereby preventing the over-reduction of the plastoquinone pool that triggers oxidative stress [28]. Similarly, and consistently we found that AbrB2 negatively regulates strongly (FC = 8.48) the *norB* gene (*sll0450*) encoding the anti-oxidant enzymes nitric oxide reductase which uses electrons provided by quinols to protect *Synechocystis* from nitric oxide [29]. The increased expression of the *cydAB* and *norB* genes of our *abrB2*-deleted mutant is consistent with its increased resistance to oxidative stress [17], and with the proposition that hydrogen production prevents oxidative stress in behaving as an electron valve [14, 30].

3.6. AbrB2 is a master regulator that regulates (mostly negatively) various transcription regulators

In *Synechocystis*, sensor histidine kinases (Hik) and response regulators (Rre) are involved in several cellular processes as heat shock stress (Hik34), osmotic stress and high light stress (Hik33), carbon assimilation and metabolism (Hik8, LexA, Rre37), hydrogen production (LexA, AbrB1, AbrB2), protection against oxidative stress and metal stresses (Slr1738) or regulation of alcohol dehydrogenase *adhA* (Rre1) [10, 18, 31-37]. We found that AbrB2 negatively regulates 12 regulator genes: *sll1292* (Rre11), *sll1291* (Rre12), *slr1305* (Rre41), *slr0210* (Hik9), *slr0311* (Hik29), *sll1872* (LytR), *slr0449* (Dnr), *sll1161* (Cya3), *slr1860* (IcfG), *slr1861, slr0846* (Rrf2) and *slr1245* the protein belonging to the LysR family of transcription regulators. By contrast, AbrB2 positively regulates the *slr1594* (Rre5) regulator gene, and it regulates neither LexA nor AbrB1 the positive regulators of the *hox* operon. Together, the absence of AbrB2 regulation of LexA and AbrB1, and our recent findings that AbrB2 negatively regulates the *hox* operon through binding to its promoter [17] demonstrate that the AbrB2-mediated repression of the *hox* operon is entirely achieved through the binding of AbrB2 onto the *hox* operon promoter, i.e. it does not involve a negative effect of AbrB2 on the expression of the other Hox regulators LexA and AbrB1. The finding that AbrB2 regulates the divergently transcribed opposite genes *norB* (*sll0450*, see above) and *dnr* (*slr0449*) [38], is not unprecedented. Indeed, we recently reported that two opposite genes could be regulated the same way (also negatively) by the same transcription factor [39].

Concerning the AbrB2 down-regulated genes *slr1861* and *slr1860*, which encode the Slr1861 kinase phosphorylating the Slr1856 and Slr1859 proteins and the Slr1860 phosphatase dephosphorylating Slr1856 [40], we noticed with great attention that these four genes belongs to the same continuous region of the chromosome carrying 26 genes all negatively regulated by AbrB2 (Fig. 2). Furthermore, these genes are interesting in that they constitute four large clusters (possibly four operons): (i) *slr1406* to *slr1410*, (ii) *sll1307* to *sll1783*, (iii) *slr1852* to *slr1862* and (iv) *slr1863* to *slr1866*, and a large number of them (*slr1406, slr1409, slr1410, sll1307, sll1306, sll1785, sll1784*) may encode periplasmic proteins possibly involved in the AbrB2-downregulated transport systems mentioned above.

3.7. AbrB2 regulates (mostly negatively and strongly) a large number of plasmidic genes, thereby suggesting that plasmids are important for cell physiology and possibly hydrogen production too

A high proportion of the plasmidic genes appeared to be regulated, mostly negatively and often quite strongly, by AbrB2 (Table 1, and Fig. 3). This is especially true of the two largest plasmids pSYSX and pSYSM, which share many genes in common [3], and (Fig. 3). Interestingly, the AbrB2-regulated genes of the pSYSX plasmid might define a very large genes cluster duplication of which suggest that it encodes one or several functions important for the fitness of *Synechocystis*. Furthermore, together with our findings that AbrB2 represses the *hox* operon encoding the hydrogenase enzyme, our present data suggest that some of the AbrB2-regulated plasmidic genes might somehow operate in the production of hydrogen. This hypothesis is presently tested in our laboratory.

4. CONCLUSION

We report on the development of truly pan-genomic (oligonucleotide) DNA microarray to study the genome-wide transcriptome responses of the best-characterized cyanobacterium *Synechocystis* to environmental stresses and/or to

reprogramming for the production of biofuels. In addition we have developed SVGMapping an R package to facilitate the analysis of the large number of *omics* data and visualize them onto custom-made templates depicting genome organization, metabolic pathways and cellular structures. Using these tools, we characterized and compared the transcriptome profiles of our *ΔabrB2* deletion mutant, with an improved hydrogen production [17], and of the WT strain. We show here that AbrB2 is a master regulator that regulates (mostly negatively) a large number of chromosomal genes operating in hydrogen production, regulation, metal transport and protection against oxidative stress, as well as numerous plasmid genes of as yet unknown function. These findings suggest that the regulation of hydrogen production might be rather complex, an hypothesis currently tested in our laboratory, and that plasmid encoded functions, which have been overlooked so far, are involved in hydrogen production, among other important cellular processes mentioned above. Consequently, we believe that the present report will stimulate both basic and applied researches on plasmid functions and their likely relation with hydrogen production.

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